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Original Article

Telmisartan suppresses cardiomyocyte and alveolar wall hypertrophy by the PPARγ-ERK-NFAT complex by changing the balance of PPARγ and ERK

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Abstract: Telmisartan inhibits cardiomyocytes by activating peroxisome proliferator-activated receptor (PPARγ), downregulating extracellular signal-regulated kinase (ERK), and inhibiting nuclear factor of activated T cells (NFAT). However, it has been unclear whether telmisartan is intrinsically associated with PPARγ, ERK, and NFAT. The present study focused on the role of telmisartan with respect to PPARγ, ERK, and NFAT. Angiotensin II was used to stimulate primary cardiomyocytes to create a cardiomyocyte hypertrophy model *in vitro* with increased pathologic protein synthesis and NFAT nuclear translocation. Telmisartan suppressed angiotensin II-induced cardiomyocyte hypertrophy by inhibiting protein synthesis and NFAT nuclear translocation. The inhibition by telmisartan was reversed by both a PPARγ inhibitor and ERK activator. These results indicated that PPARγ and ERK play opposing roles in regulating telmisartan inhibition of cardiomyocyte hypertrophy. When we precipitated cardiomyocyte NFAT, we found that PPARγ and ERK bind to NFAT, indicating that the PPARγ-ERK-NFAT complex mediated telmisartan inhibition of cardiomyocyte hypertrophy. In this complex, the balance of PPARγ and ERK is critical to regulate NFAT function. Finally, we created a new model to explain the mechanism by which telmisartan prevents cardiomyocyte hypertrophy.

Keywords: Telmisartan, cardiomyocyte hypertrophy, NFAT, PPARγ, ERK

Introduction

Cardiac hypertrophy is an adaptive response and a common complication to various pathological stimuli, such as hypertension and myocardial infarction [1, 2]. Sustained cardiac hypertrophy leads to the maladaptive response known as heart failure, with enlarged ventricular volume and adynamic contractility. Cardiomyocyte hypertrophy as an original pathological response is always followed by extracellular hypertrophic signal stimulation, intracellular signal transduction, and genetic transcription and activation, which ultimately induces cellular hypertrophy accompanied by abnormal protein synthesis.

Telmisartan, a well-known angiotensin II receptor blocker, selectively blocks the angiotensin II receptor [3]. Many studies have reported that telmisartan inhibits left ventricular hypertrophy and improves cardiac function [4, 5]. However, the mechanism by which telmisartan inhibits cardiomyocyte hypertrophy is unclear. Telmisartan improves left ventricular remodeling of infarcted hearts by activating peroxisome proliferator-activated receptor γ (PPARγ) [6] and inhibiting extracellular signal-regulated kinase (ERK) phosphorylation [7]. However, more details are needed to clarify the crosstalk between PPARγ and ERK in the inhibition of cardiomyocyte hypertrophy.

PPARs are transcription factors of the nuclear hormone receptor superfamily that bind to specific DNA sequences [8]. PPARs form heterodimers with RXR to regulate various genes, and they exist as three subtypes (α, β/δ, γ) with related structures [9]. PPARγ, also known as NR1C3 (nuclear receptor subfamily 1, group C, member 3), the most studied subtype, is involved in lipid metabolism, adipocyte differentiation, and adipogenesis as well as glucose metabolism [10]. Clinically, PPARγ agonists have been used in the treatment of hyperlipidemia and hyperglycemia [11]. PPARγ inhibits inflammatory responses in cardiovascular cells, including cardiomyocytes [12]. Hyperlipidemia, hyperglycemia, and inflammation are all key factors that induce cardiomyocyte hypertrophy or heart failure. Recently, it was reported that PPARγ activation by an agonist plays an important role in inhibiting cardiomyocyte hypertrophy via nuclear factor of activated T cells C4 (NFATc4) nuclear translocation [13], and that PPARγ physically associates with transcriptional factor NFAT to block NFAT DNA binding and transcriptional activity [14]. This seemed to provide a direct mechanism for PPARγ activity beyond affecting metabolism and inflammation, and may indicate the existence of crosstalk between them.

ERK is also a well-known transcription factor that leads to cardiomyocyte hypertrophy [15]. Inhibition of ERK phosphorylation is another potential mechanism for suppressing cardiomyocyte hypertrophy via telmisartan [7]. ERK-induced cardiomyocyte hypertrophy plays an additional role by forming an ERK-NFAT complex [16]. Recently, it was reported that telmisartan suppresses cardiac hypertrophy by inhibiting cardiomyocyte apoptosis via the NFAT/ANP/BNP signaling pathway [17]. Both PPARγ and ERK associate with NFAT, which is a critical factor that is induced during pathological cardiomyocyte hypertrophy [18]. However, the precise interactions by which PPARγ, ERK, and NFAT regulate telmisartan inhibition of cardiac hypertrophy are unknown.

In the present study, we examined how the PPARγ-ERK-NFAT complex is involved in telmisartan-suppressed cardiomyocyte hypertrophy. The balance of PPARγ and ERK may be very important in regulating cardiomyocyte hypertrophy by telmisartan. We also found that the PPARγ-ERK-NFAT complex is involved in the suppressive effect of telmisartan on alveolar wall hypertrophy, which further indicated that the PPARγ-ERK-NFAT complex mediates the role of telmisartan. This may not be limited only to heart tissue, but may be a universal mechanism.

Materials and methods

Cell culture of primary rat cardiomyocytes

One or two-day-old neonatal Wistar rats were purchased from the Experimental Animal Center of the Academy of Military Medical Sciences, Beijing, China. Rat hearts were washed to remove blood in sterilized phosphate buffered saline (PBS) three times after routine harvesting under aseptic conditions. Hearts were then minced in a sterilized small bowl and digested in cardiomyocyte balance suspension liquid with 0.1% trypsin and 0.025% collagenase for 15 min. Undigested heart tissue was re-digested and the procedure repeated four times until all tissues were digested. Cardiomyocytes were separated and concentrated by centrifugation within a Percoll gradient system. The cardiomyocytes were cultured in Dulbecco's Modified Eagle's Medium (DMEM) with 10% fetal bovine serum (FBS) and penicillin at a density of $1 \times 10^5/cm^2$ in a 5% CO_2 incubator at 37°C. Cardiomyocytes were used in various experiments from the second day. This study was approved by the Medical Ethics Committee of Heilongjiang Provincial Hospital, Harbin, China.

[^{3}H] leucine incorporation

Cardiomyocytes were pretreated with 1 μM GW9662, a PPARγ inhibitor (Sigma-Aldrich, Santa Clara, CA, USA) [19], or 20 nM 12-0-tetradecanoylphorbol-13-acetate (TPA), an ERK activator (Cell Signaling Technology, Inc., Shanghai, China) [20], for 30 min and then treated with 50 μM telmisartan (Boehringer Ingelheim, Shanghai, China) in the presence or absence of 10 μm angiotensin II (Sigma-Aldrich) for 18 h. After incubation with [^{3}H] leucine (1 μCi/ml) for 12 h, the plates were washed with ice-cold PBS three times. Then, after incubation with ice-cold trichloroacetic acid (5% v/v) at 4°C for 1 h, they were again washed with ice-cold PBS three times. Cells were then dissolved in 0.1 mol/l NaOH. Liquid scintillation counting was used to measure the radioactivity.

Immunofluorescence and confocal microscopy

Cardiomyocytes were collected and grown on glass-bottom μ-slides in four-well dishes (Thundscience, Shanghai, China) precoated with collagen II. After being treated with reagents, cells were fixed with acetone. Double immunofluorescent staining was performed using a specific antibody against NFATc4 (1:800, sc-13036, Santa Cruz, CA, USA) and donkey anti-rabbit IgG (red) (Alexa Fluor® 488, Abcam, Shanghai, China) or Myh6 (1:500, sc-167686, Santa Cruz) and donkey anti-goat IgG (red). Finally, slides were mounted in fluorescence mounting medium with DAPI (4',6 diamidio-2-phenylindole) (blue) (Vector Laboratories, Shanghai, China). Cells were observed by an Olympus FluoView™ FV1000 fluorescence microscope.

Western blot

Cytoplasmic protein or nuclear protein was extracted using lysis buffer (50 mM Tris·HCl, pH 7.5, containing 150 mM NaCl, 25 mM EDTA, 0.25% sodium deoxycholate, and 1 mM DTT) or RIPA buffer (50 mM Tris·HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1% NP-40) as reported previously [17]. Briefly, whole cell pellets were dissolved using lysis buffer, then the supernatant was carefully extracted as the cytoplasmic protein fraction after high speed centrifugation (15,000 rpm). The remaining precipitate was dissolved using RIPA buffer, then the supernatant was extracted as the nuclear protein fraction after a second centrifugation. The protein concentration was determined using a BCA Protein Assay Kit (Pierce, 23225, Thermo Fisher Scientific, Shanghai, China). The proteins (10 μg) were electrophoresed by SDS-PAGE (NP0301BOX, Invitrogen, Shanghai, China) and subsequently transferred to a PVDF membrane (IPVH00010, Millipore, Shanghai, China) that was blocked in TBST containing 5% skim milk for 60 min at room temperature. After incubation with antibodies against NFATc4 (1:1000, sc-13036, Santa Cruz) or PPARγ (1:1000, sc-6285, Santa Cruz) overnight at 4°C, the membrane was incubated with peroxidase-conjugated secondary rabbit or goat IgG antibody (Sigma-Aldrich). Blots were incubated with ECL Western Blotting Detection Reagent (GE Healthcare Life Science), visualized by using a Luminescent Image Analyzer LAS-3000 system (Fujifilm, Tokyo, Japan) and quantified by Image J software (National Institutes of Health, NIH).

Co-immunoprecipitation

NFATc4 was immunoprecipitated by incubated cellular extracts (150 μg protein) with an anti-NFATc4 antibody (sc-13036, Santa Cruz), and 20 μl protein A agarose bead slurry was added to react overnight at 4°C on a rocking platform. Protein A agarose beads were collected from the reaction solution by centrifugation at 6,000 rpm for 5 min and washed three times (5 min per wash) with PBS. After removing the supernatant, 10 μl sample buffer was added and boiled at 100°C for 3 min. All 10 μl of the resulting liquid sample was subjected to SDS-PAGE followed by normal western blotting procedure.

Measurement of cardiomyocyte volume

Cardiomyocyte area was measured randomly by Image J software (National Institutes of Health, NIH). Single or sporadic cells were selected for measurement. About 50 cells/visual field for five fields were measured to obtain the average area per well.

RT-PCR

Total RNA was extracted from hearts using TRIzol (Invitrogen, Carlsbad, CA, USA), and cDNA (50 ng/μl) was synthesized by using oligo (dT) primers with the Transcriptor First Strand cDNA Synthesis Kit (04896866001, Roche, Shanghai, China). The PCR amplifications were quantified by using SYBR Green (04887352001, Roche). The results were normalized by GAPDH rRNA gene expression. The primers used were forward 5'-AGATCATCAAGGCCAAGGCA-3', reverse 5'-CGCTGGGTGGTGAAATCATT-3' for Myh6; forward 5'-TCGCGACCTTACTGACTACCTG-3', reverse 5'-GCTTCTCTTTGATGTCGCGC-3' for Acta1; forward 5'-GTGAAGGTCGGAGTCAACG-3', reverse 5'-GGTGAAGACGCCAGTGGACTC-3' for GAPDH.

Telmisartan administration and HE staining

Mice were purchased from Heze Better Biotechnology, Shandong, China, and had free access to normal chow diet and water. Temperature and humidity were kept at 24°C, 50-60% with a 12 h light/12 h dark cycle. Male C57BL/6 mice weighing 24-26 g were used throughout the experiment and randomly divided into four groups for vein/subcutaneous or intra-tracheal administration under intraperitoneal anesthesia (pentobarbital salt, 50 mg/kg). Cardiomyocyte inhibition experiments were performed as reported previously [17]. For one group, GW9662 (100 μm mouse) and TPA (100 nM/mouse) were injected in the tail vein three times each for 2 days before telmisartan administration. For pulmonary inhibition experiments, three groups were given intra-tracheal adminis-

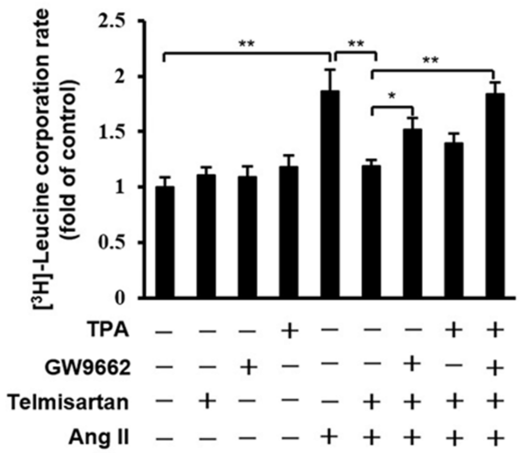

Figure 1. PPARγ and ERK are involved in telmisartan inhibition of Ang II-induced [3H] leucine incorporation in primary cultured cardiomyocytes. Cardiomyocytes were pretreated with 1 μm GW9662 or 20 nM 12-0-tetradecanoylphorbol-13-acetate (TPA) for 30 min followed by stimulation with 50 μM telmisartan in the presence or absence of 10 μm Ang II for 18 h. Cardiomyocytes were incubated with [3H] leucine (1 μCi/ml) for 12 h. [3H] leucine-related protein incorporation was determined by liquid scintillation counting. (Data are the means ± SEM. n = 4-6 for each group. $*P < 0.05$; $**P < 0.01$).

tration of lipopolysaccharide (LPS) (50 μg/mouse, once every 2 days for four times), and the control group was given intra-tracheal administration of PBS. Three days before LPS administration, the LPS-administration groups began administration of telmisartan through oral gavage at a dose of 50 mg/kg/day (two groups) or the same volume of saline (one group) as in a previous study [17]. One telmisartan-administration group was given intra-tracheal administration of GW9662 (100 μm/mouse) and TPA (100 nM/mouse) one day before LPS administration (once every 2 days for four times). Two weeks later in both experiments, mice were sacrificed and lung tissues were fixed with 4% formaldehyde for 16 h at room temperature. After 2 days of dehydration by ethyl alcohol (from 70% to 100%), lung samples were embedded in paraffin. Blocks were cut into 5-μm-thick sections, dewaxed in xylene for 10 min, and stained with hematoxylin and eosin (H&E) at room temperature as previously reported [17].

Statistical analysis

Results are expressed as the mean values ± SEM. Each experiment was repeated three times independently. Comparisons between groups were carried out by one-way analysis of variance. A value of $P < 0.05$ was considered significant.

Results

PPARγ and ERK are involved in telmisartan inhibition of protein synthesis

Cardiomyocyte hypertrophy is always accompanied by pathological protein synthesis. To understand whether telmisartan could inhibit abnormal protein synthesis and whether PPARγ and ERK were involved, we first measured protein synthesis under various conditions. As shown in **Figure 1**, angiotensin II (Ang II) induced pathological protein synthesis by 1.8-fold compared to the control, and this was almost completely inhibited by telmisartan treatment. This inhibition was partly attenuated by either a PPARγ inhibitor (GW9662) or ERK activator (TPA), and interestingly, the inhibition was almost completely attenuated by a PPARγ inhibitor and ERK activator in combination (**Figure 1**). However, these effects were not found in cardiomyocytes without Ang II pretreatment (**Figure 1**), which means that telmisartan does not affect normal cardiomyocytes but only Ang II-induced hypertrophied cardiomyocytes. These results indicated that both PPARγ and ERK are involved in telmisartan inhibition of cardiomyocyte pathological protein synthesis, though with opposite roles.

PPARγ and ERK mediate telmisartan inhibition of cardiomyocyte hypertrophy

Since telmisartan inhibits left ventricular hypertrophy and improves cardiac function [4, 5], we next asked whether PPARγ and ERK also mediate improved cardiomyocyte hypertrophy by telmisartan. To test this hypothesis, we first investigated cardiomyocyte volume by observing live and Myh6-stained cultured cardiomyocytes (**Figure 2A**, **2B**). As a result, Ang II induced cardiomyocyte hypertrophy by 2-fold compared to the control group, and telmisartan strongly inhibited Ang II-induced cardiomyocyte hypertrophy. However, the hypertrophy inhibition effect of telmisartan was negated by a combination of the PPARγ inhibitor GW9662 and the ERK activator TPA (**Figure 2A**, **2B**). To verify this result, cardiomyocyte hypertrophy-associated markers (*Myh6* and *Acta1*) were also measured. Telmisartan inhibited *Myh6* and *Acta1* mRNA expression induced by Ang II. This inhibition was also blocked by the GW9662 and TPA

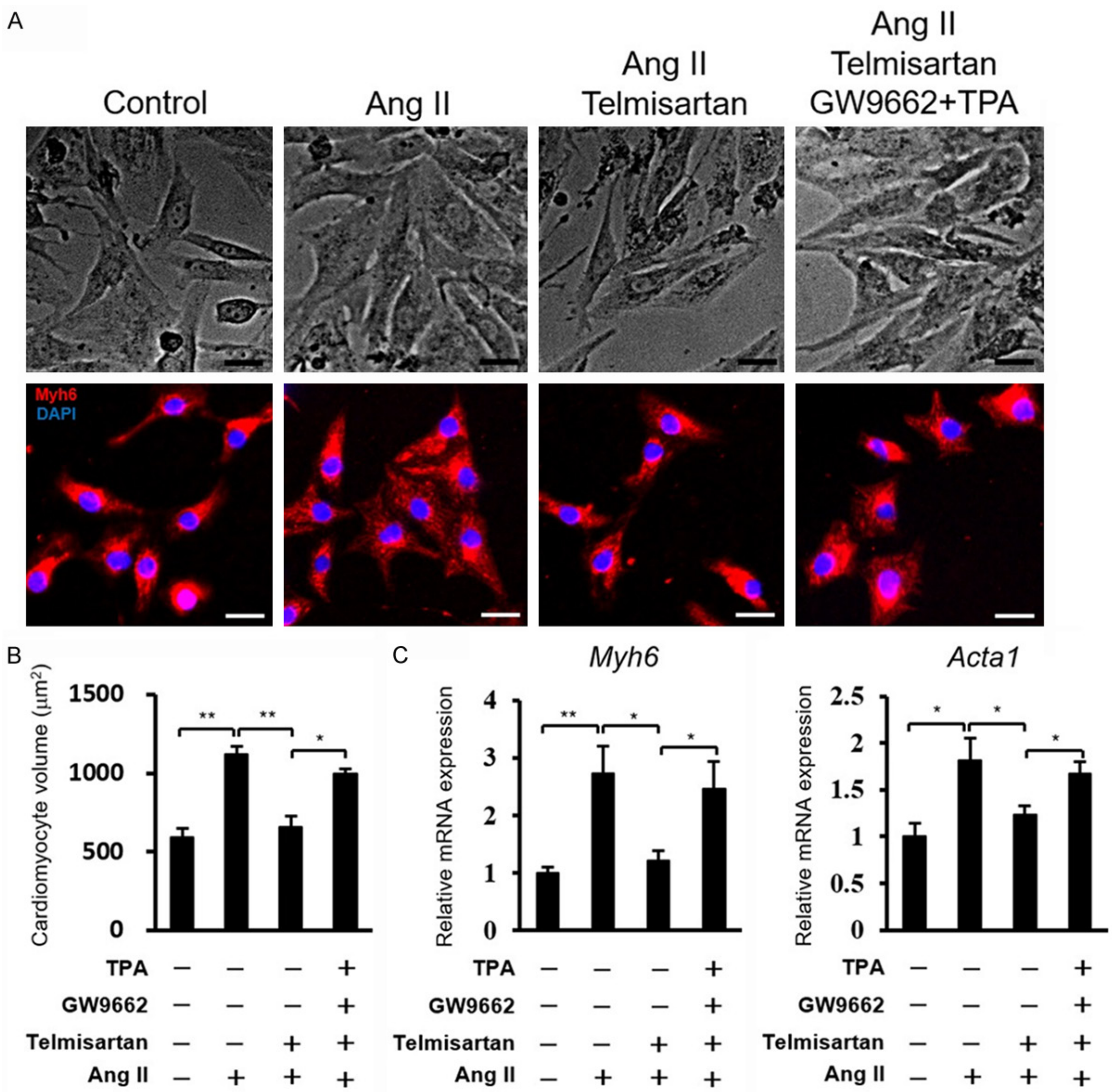

Figure 2. PPARγ and ERK are involved in telmisartan inhibition of Ang II-induced cardiomyocyte hypertrophy. (A) Representative images of live cardiomyocytes after telmisartan or GW9662 and TPA treatment with or without Ang II stimulation (upper). Representative immunofluorescent staining of cardiomyocytes by Myh6 (red) and DAPI (blue) (below). Scale bars = 50 μm. (B) Calculation of cardiomyocyte volume from (A). (C) *Myh6* and *Acta1* mRNA expressions in cardiomyocytes were quantified by RT-PCR as an additional index for cardiomyocyte hypertrophy. (Data are the means ± SEM. n = 5 for each group. *$P < 0.05$, **$P < 0.01$).

combination (**Figure 2C**). These results indicated that PPARγ and ERK mediated the cardiomyocyte hypertrophy inhibition effect of telmisartan and are consistent with the results shown in **Figure 1**.

PPARγ and ERK are involved in inhibition of NFAT nuclear translocation induced by telmisartan

NFAT is a key factor in inducing cardiomyocyte hypertrophy by nuclear translocation [18], and both PPARγ and ERK have been reported to be functionally associated with NFAT [14, 16]. We therefore examined whether telmisartan inhibited NFAT nuclear translocation and the roles of PPARγ and ERK in this process. In primary cardiomyocytes, Ang II induced significant NFATc4 nuclear translocation, which was inhibited by telmisartan. Interestingly, this inhibition was impaired by the PPARγ ginhibitor and ERK activator combination (**Figure 3A**, **3B**). An additional experiment was also performed to verify this inhibition. Western blotting showed that upregulated nuclear NFATc4 translocation induced

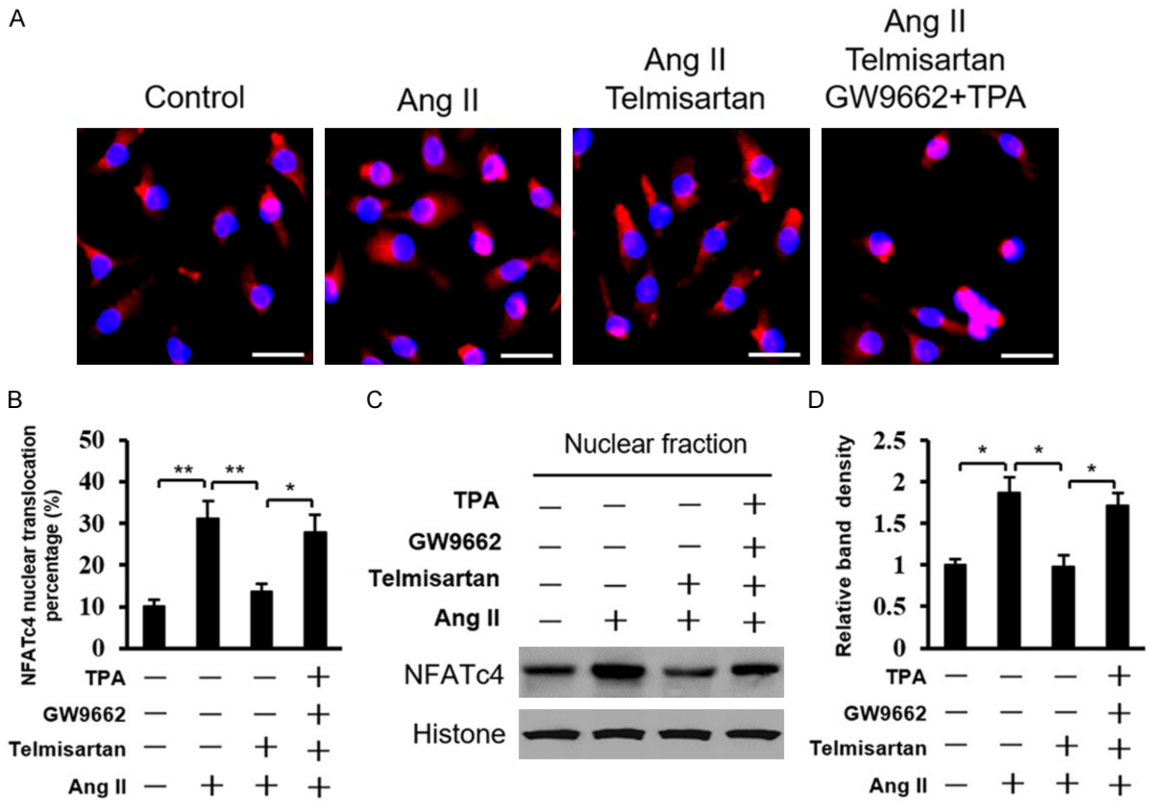

Figure 3. PPARγ and ERK are involved in telmisartan inhibition of Ang II-induced NFAT nuclear translocation in primary cultured cardiomyocytes. (A) Representative immunofluorescent staining of cardiomyocytes with NFATc4 (red) and DAPI (blue) after telmisartan or GW9662 and TPA treatment with or without Ang II stimulation. Scale bars = 50 μm. (B) Quantification of (A). (C) Representative western blot of NFATc4 after telmisartan or GW9662 and TPA treatment with or without Ang II stimulation. Histone was used as an internal control for loading protein volume. (D) Relative quantification of (C). (Data are the means ± SEM. n = 5 for each group of (B). n = 3-4 for each group of (C). **P* < 0.05; ***P* < 0.01).

by Ang II was inhibited by telmisartan, and this inhibition was impaired by the PPARγ inhibitor and ERK activator combination (**Figure 3C**, **3D**). These results indicated that both PPARγ and ERK are involved in the inhibition of NFATc4 nuclear translocation by telmisartan.

The PPARγ-ERK-NFAT complex regulates NFAT inhibition by telmisartan

The above results showed that PPARγ activation and ERK inactivation facilitate NFAT function. It seems that PPARγ and ERK play opposite roles in this regulation. To understand how these opposing roles are regulated by the two molecules, we precipitated cardiomyocyte NFATc4 under Ang II stimulation and found that both PPARγ and ERK combined with NFATc4. Telmisartan treatment increased the binding with PPARγ and decreased that with ERK simultaneously (**Figure 4A**, **4B**). As a result, telmisartan changed the PPARγ/ERK ratio and this was prevented by PPARγ inhibitor and ERK activator (**Figure 4C**). These results indicated that PPARγ and ERK regulate NFAT by interacting with it directly. The balance of PPARγ and ERK may therefore directly decide the fate and function of NFAT.

The PPARγ-ERK-NFAT complex is involved in telmisartan inhibition of cardiomyocyte hypertrophy in mouse heart

Next, we tested whether telmisartan regulation of the PPARγ-ERK-NFAT complex also occurred in the heart *in vivo* by administering telmisartan with or without the PPARγ inhibitor and ERK activator to Ang II-induced cardiac hypertrophy mice. We found that telmisartan suppressed Ang II-induced cardiac hypertrophy of mice, the same result as reported previously [17], and that the PPARγ inhibitor and ERK activator

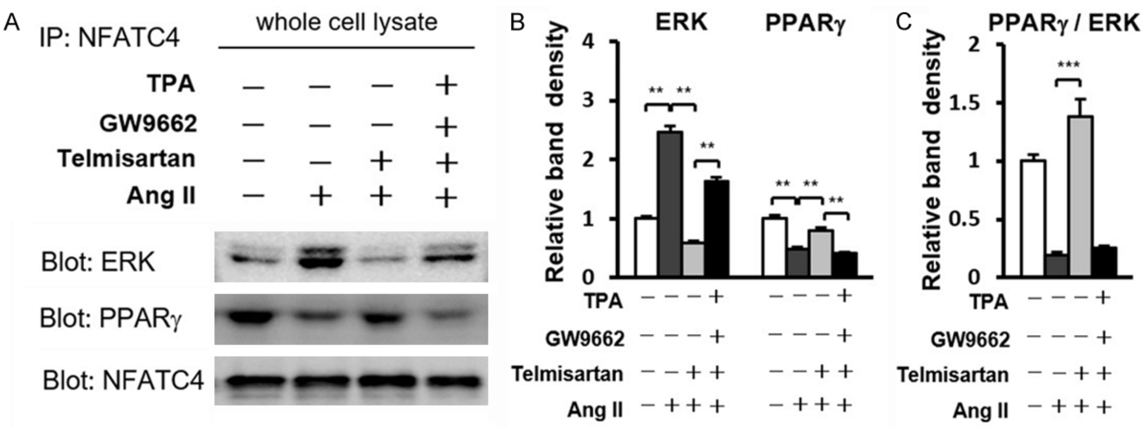

Figure 4. PPARγ and ERK regulate NFATc4 in cardiomyocytes by forming a PPARγ-ERK-NFAT complex. (A) Representative western blot of ERK and PPARγ in immunoprecipitation of NFATc4 from cardiomyocytes after Ang II treatment. (B) Quantification of ERK and PPARγ in (A). (C) Relative quantification of the PPARγ/ERK ratio in (A). (Data are the means ± SEM. n = 5 for each group. *$P < 0.05$).

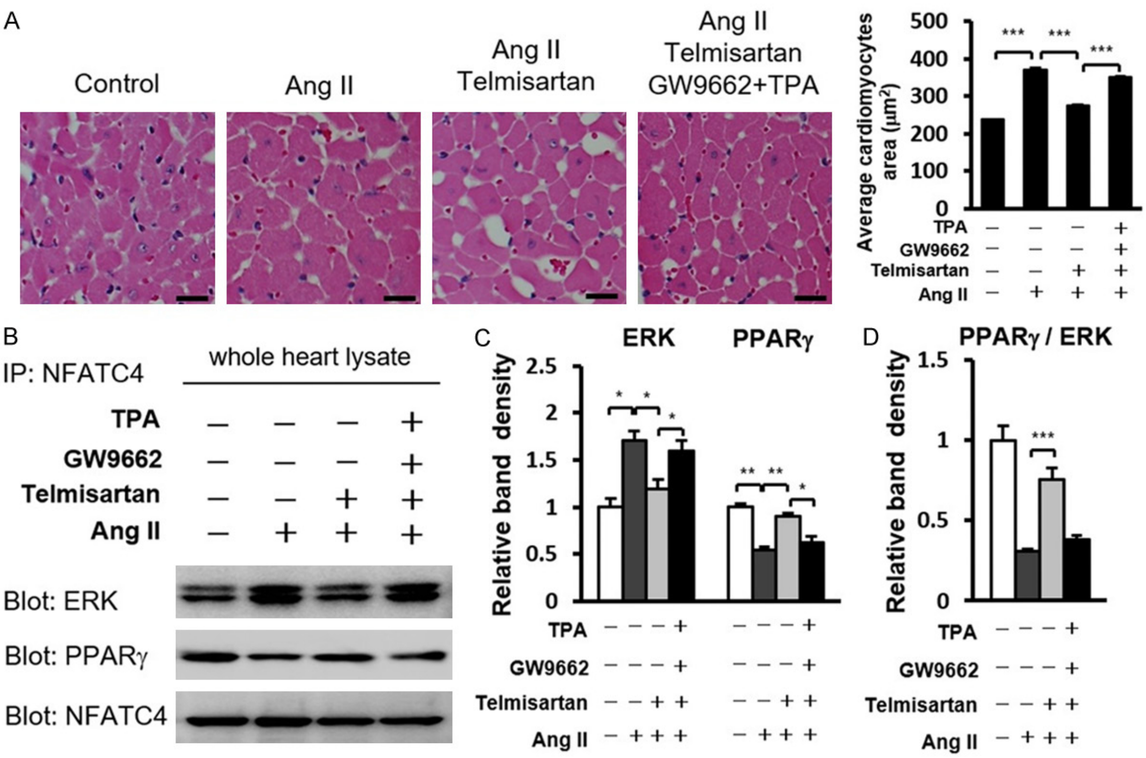

Figure 5. The PPARγ-ERK-NFAT complex mediates cardiac hypertrophy in mouse heart. (A) Representative image of heart tissue with H&E staining and average cardiac myocyte area quantification after telmisartan or GW9662 and TPA subcutaneous administration with or without Ang II pre-stimulation. Scale bars = 20 μm. (B) Representative western blot of ERK and PPARγ in immunoprecipitated NFATc4 in heart tissue. (C) Relative quantification of ERK and PPARγ in (B). (D) Relative quantification of the PPARγ/ERK ratio in (B). (Data are the means ± SEM. n = 4 for each group. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$).

blocked the role of telmisartan (**Figure 5A**). When we precipitated cardiac NFATc4, we observed that telmisartan increased the amount of bound PPARγ and decreased bound ERK, thus increasing the PPARγ/ERK ratio (**Figure 5B-D**), and the PPARγ inhibitor and ERK

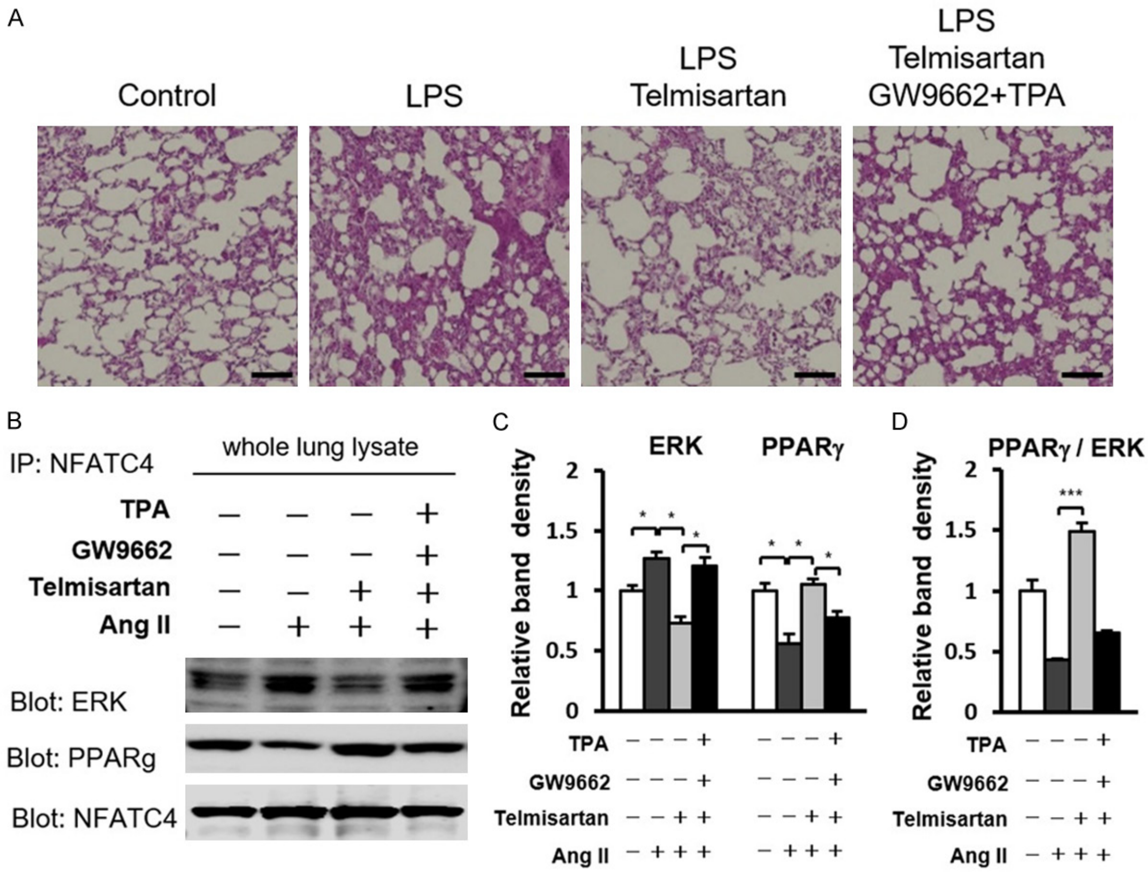

Figure 6. The PPARγ-ERK-NFAT complex mediates alveolar wall hypertrophy. (A) Representative image of lung tissue with H&E staining after telmisartan or GW9662 and TPA tracheal administration with or without LPS pre-stimulation. Scale bars = 100 μm. (B) Representative western blot of ERK and PPARγ in immunoprecipitated NFATc4 of lung tissue. (C) Relative quantification of ERK and PPARγ in (B). (D) Relative quantification of the PPARγ/ERK ratio in (B). (Data are the means ± SEM. n = 4-6 for each group. *P < 0.05; **P < 0.01).

activator prevented this (**Figure 5B**, **5C**). These results are similar to those in cultured cardiomyocytes and indicated that telmisartan regulates the PPARγ-ERK-NFAT complex and suppresses cardiac hypertrophy in mice.

The PPARγ-ERK-NFAT complex is involved in telmisartan inhibition of alveolar wall hypertrophy

Next, we tested whether the role of telmisartan via the PPARγ-ERK-NFAT complex was specific to the heart or could broadly regulate other tissues. It has been reported that NFAT promotes pulmonary inflammation [21], which is always accompanied with alveolar wall hypertrophy. Also, ERK activation induces lung inflammation [22], while PPARγ has been reported to suppress lung injury and inflammation [23]. We therefore surmised that telmisartan may also regulate the PPARγ-ERK-NFAT complex in lung tissue. To test this hypothesis, we investigated the effect of telmisartan in a lung inflammation model. A mouse pulmonary inflammation model was established by tracheal administration of LPS, which is commonly used to induce lung inflammation [24, 25]. Interestingly, telmisartan tracheal administration significantly suppressed LPS-induced alveolar wall hypertrophy. This suppression effect was blocked by PPARγ inhibitor and ERK activator administration (**Figure 6A**). Next, to demonstrate whether PPARγ and ERK also affect NFAT in the form of the PPARγ-ERK-NFAT complex within lung tissue, we precipitated pulmonary NFATc4 and found that both PPARγ and ERK bound to NFATc4. Telmisartan tracheal administration suppressed NFATc4-bound ERK and elevated NFATc4-bound PPARγ. These changes were negated by PPARγ inhibitor and ERK activator administration (**Figure 6B**, **6C**). Finally, we calculated the PPARγ/ERK, which was significantly increased after telmisartan administration (**Figure 6D**), and an identical result to what was seen in the heart.

Discussion

Telmisartan effectively prevents cardiovascular events [26]. Although PPARγ and ERK have been reported to mediate the cardiomyocyte hypertrophy inhibitory role of telmisartan [6, 7], the mechanism is not well-understood. Another cardiomyocyte critical factor, NFAT, has been reported to physically associate with PPARγ and ERK [14, 16]. In the present study, we focused on these three critical factors and explored their crosstalk in regard to telmisartan inhibition of cardiomyocyte hypertrophy.

NFAT participates in pathological, but not physiological, cardiomyocyte hypertrophy [18]. We found that NFAT nuclear translocation could be completely inhibited by telmisartan, accompanied by the complete inhibition of abnormal protein synthesis and cardiomyocyte hypertrophy. This indicated that NFAT was the central factor that plays a crucial role in the telmisartan inhibition process, more so than PPARγ and ERK. PPARγ and ERK may just be assistant factors that regulate NFAT. These results encouraged us to explore the associations between PPARγ, ERK, and NFAT.

Telmisartan inhibits NFAT nuclear translocation directly or by upregulating PPARγ [6]; however, the means by which this inhibition is mediated has been unclear. As PPARγ and ERK complex with NFAT [14, 16], and telmisartan has been reported to promote PPARγ expression and suppress ERK phosphorylation, we supposed that upregulated PPARγ and downregulated ERK participated in telmisartan-induced NFAT regulation. Based on reports of the ERK-NFAT complex and PPARγ-NFAT regulation [14, 16], we found that the PPARγ-ERK-NFAT complex was the real mediator for cardiomyocyte hypertrophy inhibition by telmisartan (**Figures 4** and **5**), and not just a single factor (PPARγ, ERK, or NFAT alone). As we discussed, NFAT is the main factor and PPARγ and ERK are assistant factors, but how the two assistant factors affect NFAT has been unknown. In the presence of Ang II, telmisartan simultaneously regulated both PPARγ and ERK, with PPARγ being upregulated and ERK downregulated. This would explain why the associations of PPARγ and ERK were parallel to telmisartan. As a result, the role of NFAT promotion mediated by ERK was blocked while the role of NFAT inhibition mediated by PPARγ was activated (**Figure 4**). This indicated that telmisartan prevented NFAT function by changing the balance of PPARγ and ERK.

For NFAT to exert its function, it requires nuclear translocation to bind with specific DNA sites to promote cardiomyocyte hypertrophy. So, we next asked how the balance of PPARγ and ERK decides NFAT function. PPARγ, a nuclear transcription factor, exerts its role within the nucleus and has been reported to block NFAT DNA binding and transcriptional activity [14]. Although ERK promotes NFAT functioning by a ERK-NFAT complex, ERK promotes NFAT-DNA binding but not NFAT nuclear translocation [16]. Both of these two previous reports seem to contradict our present study (**Figure 3**). Although we are not clear about how NFAT nuclear translocation was regulated by PPARγ and ERK, it is possible that there was a feedback between nuclear non-DNA-binding NFAT and cytoplasmic NFAT. We find this hypothesis interesting, because feedback is a universal mechanism in cellular and intercellular signaling, within organs and between organs. However, PPARγ agonists inhibit cardiomyocyte hypertrophy by the NFAT pathway [13]. Thus, it is not strange that in our present study, the imbalance of PPARγ and ERK induced by telmisartan inhibited NFATc4 nuclear translocation (**Figure 2**). However, there are four NFAT transcription factors, NFATc1 to NFATc4, each of which is expressed in cardiomyocytes [27]. Although in our present study we investigated only NFATc4, we cannot exclude similar roles of telmisartan with other NFATcs, which we will investigate in subsequent work.

Finally, we also investigated whether the role of the PPARγ-ERK-NFAT complex in mediating the effects of telmisartan was only specific to cardiomyocytes. Interestingly, we found that the PPARγ-ERK-NFAT complex also exists in lung tissue (**Figure 6**). These results demonstrated that PPARγ-ERK-NFAT complex mediation of telmisartan may be a general phenomenon, not only in cardiomyocytes, but also in lung tissues. We also cannot exclude the possibility that the

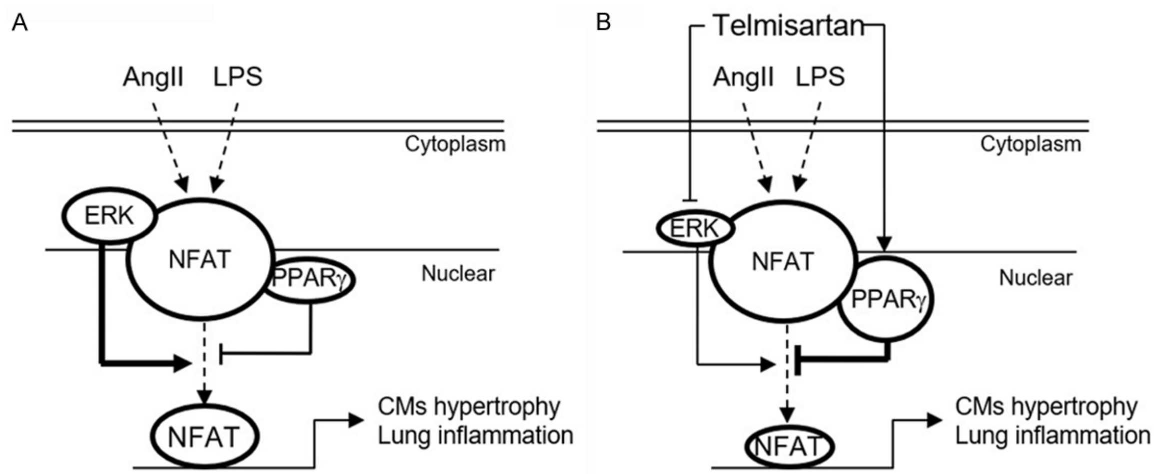

Figure 7. Schematic of a new model of telmisartan inhibition of cardiomyocyte and alveolar wall hypertrophy. A. Ang II or LPS induces cardiomyocyte or pulmonary NFAT nuclear translocation, which is co-regulated by PPARγ and ERK in the form of a PPARγ-ERK-NFAT complex. Specifically, PPARγ inhibits NFAT function and ERK promotes NFAT function by regulation of NFAT nuclear translocation. B. Telmisartan promotes PPARγ and inhibits ERK simultaneously (changing the PPARγ/ERK ratio), thus blocking NFAT nuclear translocation and DNA binding and decreasing cardiomyocyte or alveolar wall hypertrophy-related gene expression.

PPARγ-ERK-NFAT complex mediates the role of telmisartan in other organs. Telmisartan has been reported to protect against chronic kidney disease [28]. NFAT and ERK promote glomerulosclerosis by mediating podocyte apoptosis [29, 30], whereas PPARγ activation has a protective effect against glomerulosclerosis [31]. These reports at least suggest that telmisartan may protect against chronic kidney disease through the PPARγ-ERK-NFAT complex. However, there is currently no direct evidence that the PPARγ-ERK-NFAT complex mediates the role of telmisartan in other organs, so further studies are needed.

In summary, we created a new model of cardiomyocyte hypertrophy inhibition by telmisartan. As shown in **Figure 7**, in Ang II-treated cardiomyocytes or in LPS-stimulated pulmonary tissue, PPARγ and ERK balance the activation of NFAT by regulating NFAT nuclear translocation and DNA binding in the form of a PPARγ-ERK-NFAT complex (**Figure 7A**). Telmisartan simultaneously promotes PPARγ and blocks ERK, consequently regulating the balance of PPARγ and ERK induced by Ang II or LPS, and finally regulating NFAT nuclear translocation and DNA binding. As a result, cardiomyocyte hypertrophy induced by Ang II or alveolar wall hypertrophy induced by LPS are accordingly suppressed (**Figure 7B**).

Acknowledgements

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Disclosure of conflict of interest

None.

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References

[1] Sadoshima J, Xu Y, Slayter HS and Izumo S. Autocrine release of angiotensin II mediates stretch-induced hypertrophy of cardiac myocytes in vitro. Cell 1993; 75: 977-984.

[2] Messerli FH, Ventura HO, Elizardi DJ, Dunn FG and Frohlich ED. Hypertension and sudden death. Increased ventricular ectopic activity in left ventricular hypertrophy. Am J Med 1984; 77: 18-22.

[3] McClellan KJ and Markham A. Telmisartan. Drugs 1998; 56: 1039-1044.

[4] Mattioli AV, Zennaro M, Bonatti S, Bonetti L and Mattioli G. Regression of left ventricular hypertrophy and improvement of diastolic

function in hypertensive patients treated with telmisartan. Int J Cardiol 2004; 97: 383-388.
[5] Verdecchia P, Sleight P, Mancia G, Fagard R, Trimarco B, Schmieder RE, Kim JH, Jennings G, Jansky P, Chen JH, Liu L, Gao P, Probstfield J, Teo K and Yusuf S. Effects of telmisartan, ramipril, and their combination on left ventricular hypertrophy in individuals at high vascular risk in the ongoing telmisartan alone and in combination with ramipril global end point trial and the telmisartan randomized assessment study in ACE intolerant subjects with cardiovascular disease. Circulation 2009; 120: 1380-1389.
[6] Maejima Y, Okada H, Haraguchi G, Onai Y, Kosuge H, Suzuki J and Isobe M. Telmisartan, a unique ARB, improves left ventricular remodeling of infarcted heart by activating PPAR gamma. Lab Invest 2011; 91: 932-944.
[7] Chang WH. Effects of telmisartan on angiotensin II-induced cardiomyocyte hypertrophy and p-ERK1/2 phosphorylation in rat cultured cardiomyocytes. Asian Biomedicine 2011; 5: 7.
[8] Chandra V, Huang P, Hamuro Y, Raghuram S, Wang Y, Burris TP and Rastinejad F. Structure of the intact PPAR-gamma-RXR-nuclear receptor complex on DNA. Nature 2008; 456: 350-356.
[9] Greene ME, Blumberg B, McBride OW, Yi HF, Kronquist K, Kwan K, Hsieh L, Greene G and Nimer SD. Isolation of the human peroxisome proliferator activated receptor gamma cDNA: expression in hematopoietic cells and chromosomal mapping. Gene Expr 1995; 4: 281-299.
[10] Ahmadian M, Suh JM, Hah N, Liddle C, Atkins AR, Downes M and Evans RM. PPARγ signaling and metabolism: the good, the bad and the future. Nat Med 2013; 19: 557-566.
[11] Li Y, Qi Y, Huang TH, Yamahara J and Roufogalis BD. Pomegranate flower: a unique traditional antidiabetic medicine with dual PPAR-alpha/-gamma activator properties. Diabetes Obes Metab 2008; 10: 10-17.
[12] Ricote M, Huang JT, Welch JS and Glass CK. The peroxisome proliferator-activated receptor (PPARgamma) as a regulator of monocyte/macrophage function. J Leukoc Biol 1999; 66: 733-739.
[13] Bao Y, Li R, Jiang J, Cai B, Gao J, Le K, Zhang F, Chen S and Liu P. Activation of peroxisome proliferator-activated receptor gamma inhibits endothelin-1-induced cardiac hypertrophy via the calcineurin/NFAT signaling pathway. Mol Cell Biochem 2008; 317: 189-196.
[14] Yang XY, Wang LH, Chen T, Hodge DR, Resau JH, DaSilva L and Farrar WL. Activation of human T lymphocytes is inhibited by peroxisome proliferator-activated receptor gamma (PPAR-gamma) agonists. PPARgamma co-association with transcription factor NFAT. J Biol Chem 2000; 275: 4541-4544.
[15] Bueno OF, De Windt LJ, Tymitz KM, Witt SA, Kimball TR, Klevitsky R, Hewett TE, Jones SP, Lefer DJ, Peng CF, Kitsis RN and Molkentin JD. The MEK1-ERK1/2 signaling pathway promotes compensated cardiac hypertrophy in transgenic mice. EMBO J 2000; 19: 6341-6350.
[16] Sanna B, Bueno OF, Dai YS, Wilkins BJ and Molkentin JD. Direct and indirect interactions between calcineurin-NFAT and MEK1-extracellular signal-regulated kinase 1/2 signaling pathways regulate cardiac gene expression and cellular growth. Mol Cell Biol 2005; 25: 865-878.
[17] Li X, Lan Y, Wang Y, Nie M, Lu Y and Zhao E. Telmisartan suppresses cardiac hypertrophy by inhibiting cardiomyocyte apoptosis via the NFAT/ANP/BNP signaling pathway. Mol Med Rep 2017; 15: 2574-2582.
[18] Wilkins BJ, Dai YS, Bueno OF, Parsons SA, Xu J, Plank DM, Jones F, Kimball TR and Molkentin JD. Calcineurin/NFAT coupling participates in pathological, but not physiological, cardiac hypertrophy. Circ Res 2004; 94: 110-118.
[19] Starkey K, Heufelder A, Baker G, Joba W, Evans M, Davies S and Ludgate M. Peroxisome proliferator-activated receptor-gamma in thyroid eye disease: contraindication for thiazolidinedione use? J Clin Endocrinol Metab 2003; 88: 55-59.
[20] Minden A, Lin A, McMahon M, Lange-Carter C, Derijard B, Davis RJ, Johnson GL and Karin M. Differential activation of ERK and JNK mitogen-activated protein kinases by Raf-1 and MEKK. Science 1994; 266: 1719-1723.
[21] Diehl S, Krahl T, Rinaldi L, Norton R, Irvin CG and Rincon M. Inhibition of NFAT specifically in T cells prevents allergic pulmonary inflammation. J Immunol 2004; 172: 3597-3603.
[22] Lee PJ, Zhang X, Shan P, Ma B, Lee CG, Homer RJ, Zhu Z, Rincon M, Mossman BT and Elias JA. ERK1/2 mitogen-activated protein kinase selectively mediates IL-13-induced lung inflammation and remodeling in vivo. J Clin Invest 2006; 116: 163-173.
[23] Standiford TJ, Keshamouni VG and Reddy RC. Peroxisome proliferator-activated receptor-{gamma} as a regulator of lung inflammation and repair. Proc Am Thorac Soc 2005; 2: 226-231.
[24] Li H, Ruan XZ, Powis SH, Fernando R, Mon WY, Wheeler DC, Moorhead JF and Varghese Z. EPA and DHA reduce LPS-induced inflammation responses in HK-2 cells: evidence for a PPAR-gamma-dependent mechanism. Kidney Int 2005; 67: 867-874.
[25] Lin W, Wu RT, Wu T, Khor TO, Wang H and Kong AN. Sulforaphane suppressed LPS-induced in-

flammation in mouse peritoneal macrophages through Nrf2 dependent pathway. Biochem Pharmacol 2008; 76: 967-973.
[26] Yusuf S, Diener HC, Sacco RL, Cotton D, Ounpuu S, Lawton WA, Palesch Y, Martin RH, Albers GW, Bath P, Bornstein N, Chan BP, Chen ST, Cunha L, Dahlof B, De Keyser J, Donnan GA, Estol C, Gorelick P, Gu V, Hermansson K, Hilbrich L, Kaste M, Lu C, Machnig T, Pais P, Roberts R, Skvortsova V, Teal P, Toni D, Vander-Maelen C, Voigt T, Weber M and Yoon BW. Telmisartan to prevent recurrent stroke and cardiovascular events. N Engl J Med 2008; 359: 1225-1237.
[27] Wilkins BJ, De Windt LJ, Bueno OF, Braz JC, Glascock BJ, Kimball TF and Molkentin JD. Targeted disruption of NFATc3, but not NFATc4, reveals an intrinsic defect in calcineurin-mediated cardiac hypertrophic growth. Mol Cell Biol 2002; 22: 7603-7613.
[28] Nakamura T, Inoue T, Suzuki T, Kawagoe Y, Ueda Y, Koide H and Node K. Comparison of renal and vascular protective effects between telmisartan and amlodipine in hypertensive patients with chronic kidney disease with mild renal insufficiency. Hypertens Res 2008; 31: 841-850.
[29] Wang Y, Jarad G, Tripathi P, Pan M, Cunningham J, Martin DR, Liapis H, Miner JH and Chen F. Activation of NFAT signaling in podocytes causes glomerulosclerosis. J Am Soc Nephrol 2010; 21: 1657-1666.
[30] Zhang H, Ding J, Fan Q and Liu S. TRPC6 upregulation in ang II-induced podocyte apoptosis might result from ERK activation and NF-kappaB translocation. Exp Biol Med (Maywood) 2009; 234: 1029-1036.
[31] Zhou TB, Drummen GP, Jiang ZP, Long YB and Qin YH. Association of peroxisome proliferator-activated receptors/retinoic acid receptors with renal diseases. J Recept Signal Transduct Res 2013; 33: 349-352.